

Increased frequencies of CD4⁺CD25^{high} regulatory T cells in acute dengue infection

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Dengue virus infection is an increasingly important tropical disease, causing 100 million cases each year. Symptoms range from mild febrile illness to severe hemorrhagic fever. The pathogenesis is incompletely understood, but immunopathology is thought to play a part, with antibody-dependent enhancement and massive immune activation of T cells and monocytes/macrophages leading to a disproportionate production of proinflammatory cytokines. We sought to investigate whether a defective population of regulatory T cells (T reg cells) could be contributing to immunopathology in severe dengue disease.

CD4⁺CD25^{high}FoxP3⁺ T reg cells of patients with acute dengue infection of different severities showed a conventional phenotype. Unexpectedly, their capacity to suppress T cell proliferation and to secrete interleukin-10 was not altered. Moreover, T reg cells suppressed the production of vasoactive cytokines after dengue-specific stimulation. Furthermore, T reg cell frequencies and also T reg cell/effector T cell ratios were increased in patients with acute infection. A strong indication that a relative rise of T reg cell/effector T cell ratios is beneficial for disease outcome comes from patients with mild disease in which this ratio is significantly increased ($P < 0.0001$) in contrast to severe cases ($P = 0.2145$). We conclude that although T reg cells expand and function normally in acute dengue infection, their relative frequencies are insufficient to control the immunopathology of severe disease.

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Dengue infection is a major public health problem in tropical areas, predominantly in children. The mosquito-borne disease is caused by four related serotypes of the dengue virus (DV), a flavivirus like West Nile and yellow fever virus. A wide range of disease severities is observed; 100 million cases each year have dengue fever, an acute febrile illness. Half a million patients suffer from the more severe dengue haemorrhagic fever (DHF) with plasma leakage, thrombocytopenia, and occasionally hypovolemic shock (1). If untreated, the mortality rate of DHF is around 30% but can be reduced to 0.2–5% by fluid replacement. To date, no vaccines or antiviral therapies are available.

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The pathogenesis of severe dengue disease is incompletely understood. Epidemiological studies show an increased risk of severe DHF in a secondary infection with a heterologous serotype. Preexisting nonneutralizing antibodies are thought to form immunocomplexes with the virus, thereby enhancing the infection of Fcγ receptor-bearing cells like monocytes/macrophages (antibody-dependent enhancement; references 2, 3). However, it is still unclear how this can lead to plasma leakage as the major symptom of DHF. Excessive activation of the innate and adaptive immune system, especially of cross-reactive memory T cells, appears to augment the secretion of vasoactive factors like TNF-α, IFN-γ, IL-2, and IL-6 (4–6). Indeed, increased numbers of activated CD8⁺ T cells and levels of cytokines have been reported

in DHF (7, 8). Thus, the pathogenesis of dengue infection is likely to be a result of the immune response rather than of the virus itself.

CD4⁺ regulatory T cells (T reg cells) have been described to play a key role in the control of the immune system. The most commonly used marker is CD25 (IL-2R α ; reference 9), although the recently identified transcription factor FoxP3 is more specific (10). Suppression is mediated via direct cell–cell contact or via the secretion of IL-10 or TGF- β (11).

T reg cells play a role not only in autoimmunity, transplantation, and cancer but also in infections (12, 13). They can limit immunopathology, which was shown for mouse *Helicobacter hepaticus* and herpes infections, for example (14, 15). So far, they have only been described in chronic viral infections. This study is the first to report on a functional T reg cell population in a nonpersistent viral infection.

As immunopathology is thought to play a major role in the pathogenesis of severe dengue disease, we hypothesized that the T reg cell response in acute infection may be inadequate to control immune activation. However, we found that T reg cells are fully functional and expand in acute dengue infection. Moreover, they were able to suppress the production of vasoactive cytokines such as IFN- γ , TNF- α , and IL-6 in response to dengue antigens. Nevertheless, this expansion might be insufficient to control the immune response in patients with severe disease. Strong support for this hypothesis comes from our observation that the relative expansion of T reg cell/effector T cell ratios is only significant in children with mild disease in contrast to severe cases ($P < 0.0001$ and $P = 0.2145$, respectively).

RESULTS AND DISCUSSION

Characteristics of study cohort

89 serologically confirmed dengue patients were enrolled and grouped based on their disease severity (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20061381/DC1>). The acute phase of dengue infection was compared with a recovered time point. Age-matched healthy, uninfected controls were compared with the recovered samples (adults only) and showed no difference.

Identification of CD4⁺CD25^{high} T reg cells

Patients' blood cells were stained ex vivo for CD4, 8, and 25. CD4⁺ and CD8⁺ cells were gated in forward/sideward scatter plots (Fig. 1 A). CD4⁺ monocytes were excluded (Fig. 1 B). Human CD25^{high} T reg cells are not a distinct population and, therefore, are difficult to gate in a reproducible way. We observed that CD4⁺ T cells with the highest expression of CD25, which are believed to be T reg cells, show a slight reduction of CD4 expression (bend of CD4 level). As this was a consistent finding, we gated this population of cells in all samples to describe CD4⁺CD25^{high} T reg cells (Fig. 1 C).

FoxP3 staining of CD4⁺CD25^{high} T cells

As naive T cells express CD25 upon activation, the CD25^{high} cell population of acutely infected dengue patients might contain activated T cells beside T reg cells. The best marker for distinction is FoxP3 (10). To control our CD25-based gating, we stained frozen PBMCs of acutely infected and recovered subjects for FoxP3 (Fig. 1 D) or used the corresponding isotype control (not depicted) and analyzed the percentage of FoxP3⁺

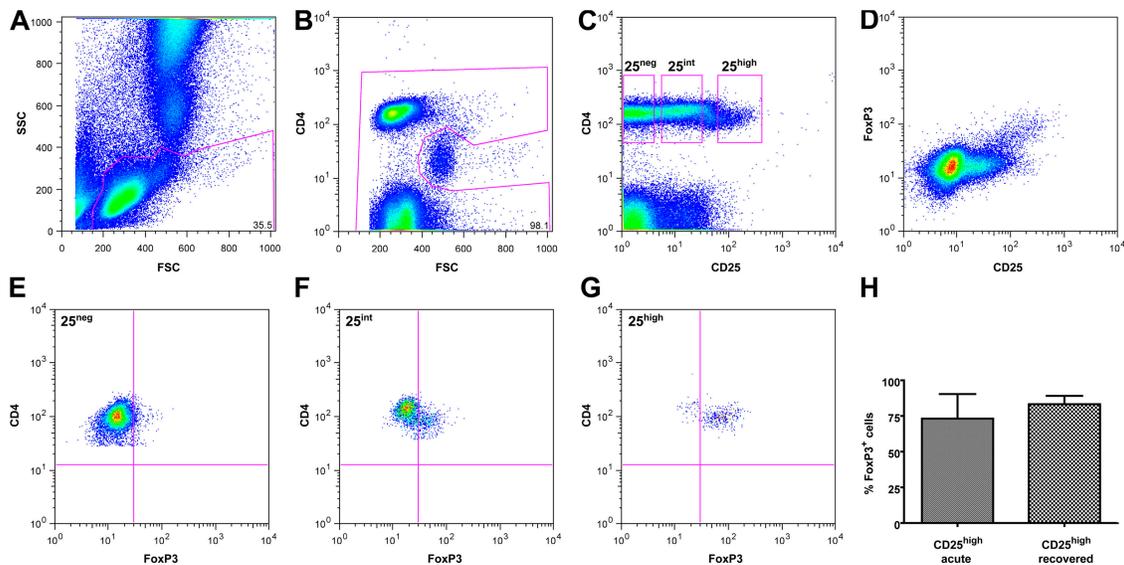


Figure 1. Identification of CD25^{high} T reg cells. Blood cells were stained ex vivo for CD4 and CD25. (A and B) Lymphocytes were gated (A), and monocytes were excluded (B). (C) CD25^{high} cells with a slightly decreased CD4 expression were gated as T reg cells. (D–G) Frozen PBMCs of acutely infected and recovered subjects were stained for CD4, CD25, and

FoxP3 (D), and FoxP3 levels of CD25^{neg}, CD25^{int}, and CD25^{high} cells were analyzed (E–G). FoxP3⁺ cells show decreased CD4 levels. (H) The percentage of FoxP3⁺ cells in the CD25^{high} population is identical at acute and recovered states. Error bars represent SEM. Applied gates are depicted as pink lines.

cells in the CD4⁺CD25^{negative} (CD25^{neg}), CD4⁺CD25^{intermediate} (CD25^{int}), and CD25^{high} gate (Fig. 1, E–G). If activated CD25⁺/FoxP3⁻ T cells fell into the CD25^{high} T reg cell gate, we would expect a drop of the percentage of FoxP3⁺ cells in acute disease in this gate. However, the percentage of FoxP3⁺ cells was comparable in acutely infected and recovered subjects (Fig. 1 H). Thus, we confirmed that even in acute dengue infection, the gated CD4⁺CD25^{high} T cells are indeed T reg cells and not activated CD25⁺ T cells. Therefore, the terms CD25^{high} cells and T reg cells will be used synonymously throughout the study.

In addition, FoxP3 analysis confirmed our observation that the CD25^{high} cells with less CD4 on their surface are human FoxP3⁺ T reg cells (Fig. 1, E–G). This reduction of CD4 expression has already been observed on mouse T reg cells (16, 17). Based on this, we suggest that a reduced CD4 expression could be used collaterally for the identification of T reg cells in cases in which FoxP3 cannot be applied as a marker.

T reg cells in dengue infection show the conventional phenotype

To describe the phenotype of T reg cells in acute dengue infection, fresh blood cells of acutely infected patients with different severities of recovered as well as uninfected donors were stained ex vivo and analyzed using flow cytometry. Expression of the memory marker CD45RO, death receptor CD95, early activation marker CD69, homing receptor CD62L, and inhibitory receptor CTLA-4 was addressed (Fig. 2, A–E). T reg

cells of uninfected donors and recovered study participants showed the same phenotype (not depicted).

At recovery, the highest percentage of CD45RO⁺, CD95⁺, CD62L⁺, or CTLA-4⁺ cells was detected in the T reg cell population. CD69 was not expressed. This is in agreement with what has been described previously in healthy donors (18, 19). Interestingly, the expression level of all of these markers was unchanged on T reg cells in acute dengue infection. Signs of activation were only detectable in the CD25^{int} gate, where, for example, CD69 and CTLA-4 expression was increased.

Collectively, the phenotype of T reg cells in acute dengue infection did not differ from that seen after recovery. This was the same for all of the different categories of disease severity. As in healthy donors, T reg cells were CD45RO^{high}, CD95^{high}, CD69⁻, CD62L^{high}, and CTLA-4^{high}. In addition, we observed that acutely activated T cells could be found in the CD25^{int} and not in the CD25^{high} gate. Based on those results together with FoxP3 analysis, we suggest that in acute dengue infection, activated T cells express lower levels of CD25 than T reg cells and can clearly be distinguished.

T reg cells suppress the proliferation of CD4⁺ T cells in acute dengue infection

We determined the functionality of T reg cells in acute dengue infection by measuring their capacity to suppress the proliferation of CD4⁺CD25⁻ responder T cells in vitro. Because

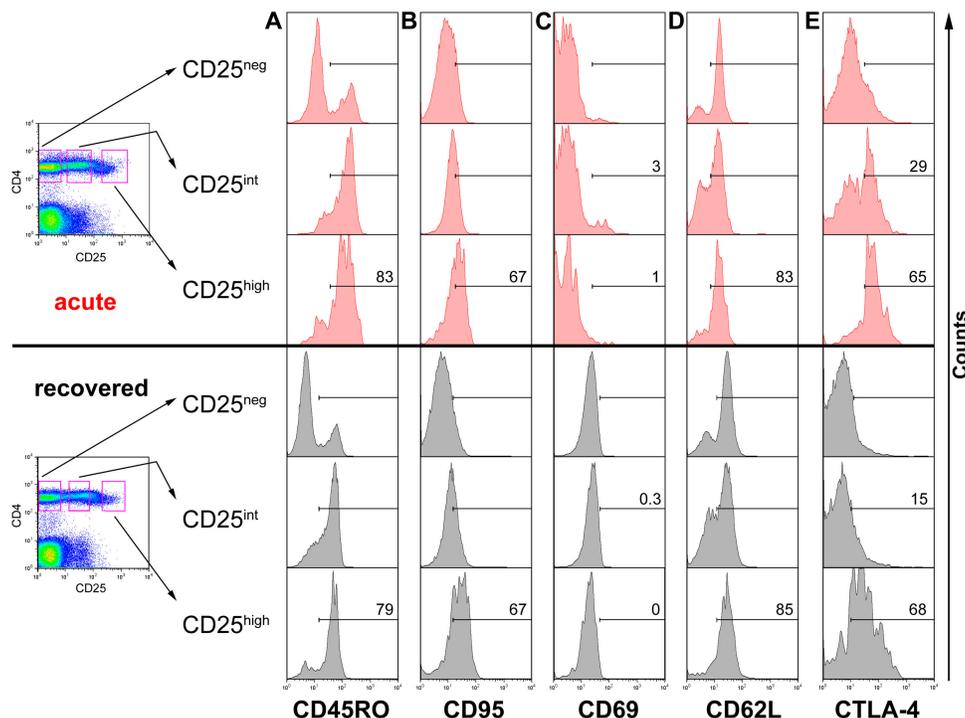


Figure 2. T reg cells in dengue infection show conventional phenotypes. (A–E) Blood cells of acutely infected ($n = 8$) and recovered ($n = 11$) subjects were stained ex vivo for CD4, 25, 45RO (A), 95 (B), 69 (C), 62L

(D), or CTLA-4 (E). CD25^{neg}, CD25^{int}, and CD25^{high} cells were compared (as indicated). Representative histograms of individual patients are shown, and numbers indicate relevant percentages of positive cells.

of the amounts of cells needed for the assay, we were restricted to samples of dengue-infected adults. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were separated from acutely infected, recovered, and uninfected subjects. The responder T cells were CFSE labeled and incubated with the same number or without T reg cells on plate-bound anti-CD3 and allogeneic APCs. After 5 d in culture, cells were analyzed by flow cytometry. As a negative control, cells were cultured without anti-CD3 (proliferation of <10%; not depicted). Analysis of the proliferation is shown for one acute sample in Fig. 3 A. Lymphocytes, including blasts, were gated and analyzed for their CFSE content. No difference was detectable between recovered study participants and uninfected donors (not depicted). The proliferative capacity of CD4⁺CD25⁻ T cells in acute dengue infection is comparable with that at convalescence: >55% of the cells proliferated (Fig. 3 B). Proliferation was reduced drastically upon the addition of T reg cells. The level of suppression was >85% in acute and recovered patients (Fig. 3 C). Reduction of the T reg cell/responder T cell ratio to 1:2 did not influence the level of suppression (not depicted). Based on these results, we conclude that T reg cells are fully functional in acute dengue infection (i.e., their suppressive capacity is not altered). We suggest that the immunopathology in dengue disease is not caused by defective T reg cell function.

Culture supernatants were collected from the suppression assays on days 1 and 5, and the IL-10 content was analyzed. Only cultures with T reg cells were substantially positive for IL-10 (Fig. 3 D). The amount of IL-10 produced was comparable in cells of both acutely infected and recovered subjects. Although IL-10 might serve as a possible mean of suppression, determination of the regulatory mechanisms needs further investigation.

T reg cells suppress the production of vasoactive cytokines after dengue-specific stimulation

The effect of T reg cells on the dengue-specific production of vasoactive cytokines was determined by IFN- γ ELISPOT. PBMCs, CD25-depleted PBMCs (CD25⁻ responder T cells), and T reg cells of 28 acute adult dengue patients were activated with DV-4 peptides plus inactivated virus (DV-2; Fig. 4 A). Stimulation of PBMCs without any, with irrelevant antigen, or of PBMCs of noninfected donors with dengue antigen (not depicted) did not result in any notable IFN- γ response. Strikingly, the IFN- γ production of dengue patients' CD25 responder T cells (CD25⁻, 1,402 \pm 635 spot-forming units/10⁶ cells) was significantly decreased (by around 50%) in the presence of T reg cells (CD25⁺, T reg cell/responder T cells \approx 1/10; P = 0.0005). In addition, even the mere depletion of T reg cells from PBMCs led to a significant increase of IFN- γ release by nearly 20% (P = 0.0186). This suggests that T reg cells are effective in suppressing cytokine production after dengue-specific stimulation at cell ratios found physiologically in the blood without the need for in vitro enrichment procedures.

To determine the effect of T reg cells on the production of other cytokines, cells of some ELISPOT assays were cul-

tured for another 48 h (Fig. 4 B). Without stimulation, no notable amounts of cytokine were detected. A significant reduction of TNF- α (P = 0.0386) by 44% was detectable in the presence of enriched T reg cells.

To analyze the effect of T reg cells on other blood cells, monocytes, CD25⁻, and T reg cells were isolated from three

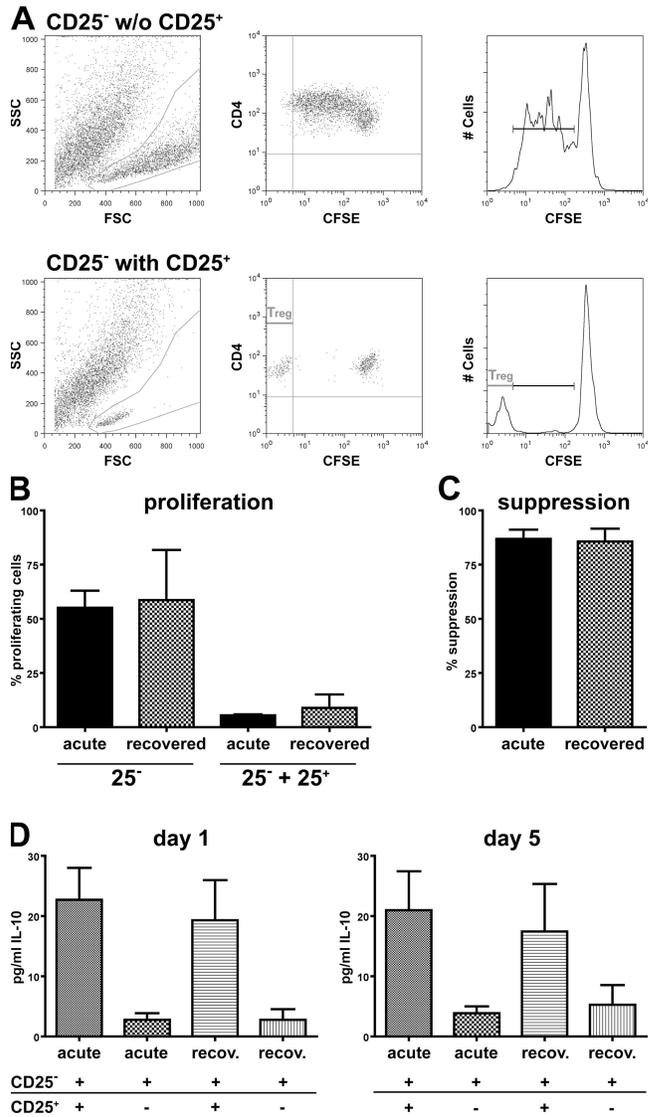


Figure 3. Suppressive capacity of T reg cells is unaltered in acute dengue infection. (A–D) CD4⁺ cells were negatively sorted from acutely infected (n = 7) and recovered (n = 3) subjects and split into CD25⁺ and CD25⁻ cells. CFSE-labeled CD25⁻ cells were incubated with or without CD25⁺ cells on plate-bound anti-CD3 and allogeneic APCs. After 5 d, proliferation was analyzed in FACS analysis. (A) A representative CFSE staining of an acutely infected patient. (B) The percentage of proliferating cells was comparable in acutely infected and recovered patients and decreased in the presence of CD25⁺ cells. (C) The suppressive capacity of the T reg cells in acute infection was like after recovery. (D) At the acute and recovered states, IL-10 was detected on days 1 and 5 in supernatants of cultures with CD25⁺ cells. Means and SEM (error bars) are shown.

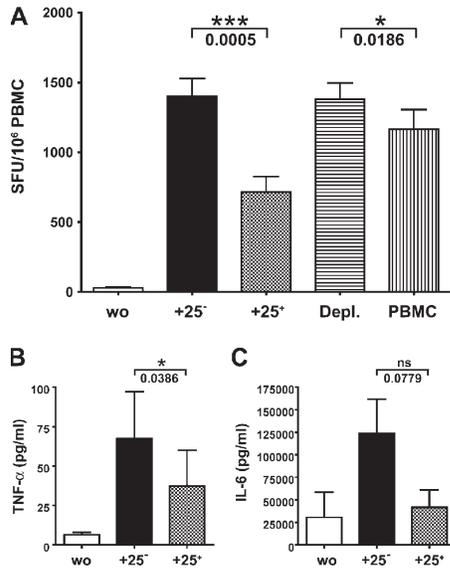


Figure 4. T reg cells suppress dengue-specific vasoactive cytokine production. (A) IFN- γ ELISPOT using CD25⁻ cells at a 10:1 ratio with CD25⁺ (+25⁺) or CD25⁻ (+25⁻) cells, PBMCs, or PBMCs depleted of CD25⁺ cells (Depl.) of 28 acute dengue patients. No peptide (wo) or DV-4 NS3 peptides and inactivated DV-2 were used as a stimulus for 12 h. In the presence of CD25⁺ cells, spot-forming units (SFU)/10⁶ cells are reduced ($n = 28$). (B) Cells of some ELISPOTs were cultured for an additional 48 h to analyze TNF- α levels in supernatants ($n = 5$). (C) After culturing monocytes of three acute patients at a 1:1 ratio with CD25⁻ (+25⁻) or CD25⁺ (+25⁺) cells with DV-4 peptides for 40 h, they were reisolated and incubated with antibody-coated inactivated DV-2 for another 48 h. IL-6 was measured in the supernatants ($n = 3$). Wo, no dengue antigens added. Means and SEM (error bars) are shown.

acute adult patients. The monocytes were cocultured at a 1:1 ratio with CD25⁻ cells or T reg cells and DV-4 peptides for T cell stimulation. After 40 h, monocytes were separated again and activated with antibody-coated inactivated DV-2 for 48 h. Subsequently, IL-6 levels were analyzed. Although the results were not significant, a trend was observed for a decrease of IL-6 production (by 66%; $P = 0.0779$) by monocytes that were precultured with T reg cells instead of CD25⁻ cells (Fig. 4 C).

From these data, we conclude that T reg cells that can suppress antigen-specific responses to dengue can be detected in the blood of acutely infected patients. As demonstrated *in vitro*, they have an impact on the expression of IFN- γ , TNF- α , and IL-6 produced by T cells and monocytes in response to dengue antigens. This is of major importance, as all of these cytokines have been linked to plasma leakage and, thus, to the severity of dengue disease (4).

T reg cell frequencies and T reg cell/effector T cell ratios are increased in acute dengue infection, especially in mild cases

The frequency of CD4⁺CD25^{high} T reg cells was determined in FACS analysis by staining patients' fresh blood cells *ex vivo*. The acute samples were collected on days 4–13 of illness. T reg cells were gated, and frequencies were analyzed as a percentage of CD4⁺ cells. Absolute CD4 counts were comparable

in severe and mild cases. In adult patients, the frequency of T reg cells at the recovered state was comparable with those of healthy controls ($2.71 \pm 0.88\%$ and $2.37 \pm 0.55\%$, respectively; not depicted). In acute infection, the T reg cell frequency was slightly increased by 32% to $3.59 \pm 1.47\%$, although it is not statistically significant (Fig. 5 A, I). A difference of 29% was detectable in children with severe disease (recovered, $3.12 \pm 1.03\%$ vs. acute, $4.01 \pm 1.3\%$; Fig. 5 A, II). With $P = 0.0531$, this increase was close to significance. However, in contrast to this, the T reg cell frequency of children with mild disease increased substantially by 61% from 2.8 ± 0.97 to $4.52 \pm 1.79\%$ (Fig. 5 A, III). This difference was highly statistically significant ($P = 0.0006$; comparable n values for severe- and mild-diseased children). Fig. 5 A (IV) summarizes the results for all three study groups.

More important than the sole increase of T reg cells for the outcome of a disease is the ratio between T reg cells and effector T cells. We determined the percentage of all CD25⁺ cells as a measure for effector T cells and calculated the T reg cell/effector T cell ratios. In both severely sick adults and children, the ratios changed in acute infection by 25% and 18%, respectively (Fig. 5 B, I and II). These differences were not statistically significant. However, in children with mild disease, the ratios increased highly significantly by 88% at the acute state ($P < 0.0001$; Fig. 5 B, III). This increase was also noted to a lower extent (38%; $P = 0.0192$) in a small group of samples collected at an early state of mild acute disease (days 1–3 of illness; not depicted). Fig. 5 B (IV) summarizes the development of the ratios for the different groups of patients.

In summary, the T reg cell population that expands in acute dengue infection has the conventional phenotype and suppressive capacity. Our demonstration that T reg cells suppress the dengue-specific secretion of vasoactive cytokines of effector T cells leads us to suggest that this population plays an active role in dengue infection. Numbers and also T reg cell/effector T cell ratios are increased. Despite this expansion of functionally intact T reg cells, patients with severe disease still develop strong immunopathology and symptoms that require hospital admission. We propose that in severe disease, the expansion of this T reg cell population is insufficient to control the immune response. This hypothesis is supported by our observation that T reg cell/effector T cell ratios are only significantly increased in children with mild and not with severe disease ($P < 0.0001$ and $P = 0.2145$, respectively; Fig. 5 B, II–IV). A change in T reg cell/effector T cell ratios could have a direct effect on the disease severity, as we demonstrated that T reg cells in the blood control the effector T cell production of vasoactive cytokines, which is directly linked to plasma leakage and disease outcome.

An alternative explanation for the relative deficiency of T reg cells in the blood of severely sick patients could be differential migration of these cells to the tissues. It has been demonstrated in HIV and hepatitis C virus infection that T reg cells can be detected at sites of preferential virus replication such as the lymphoid tissue or liver (20, 21). Although our data could be compatible with the increased migration of

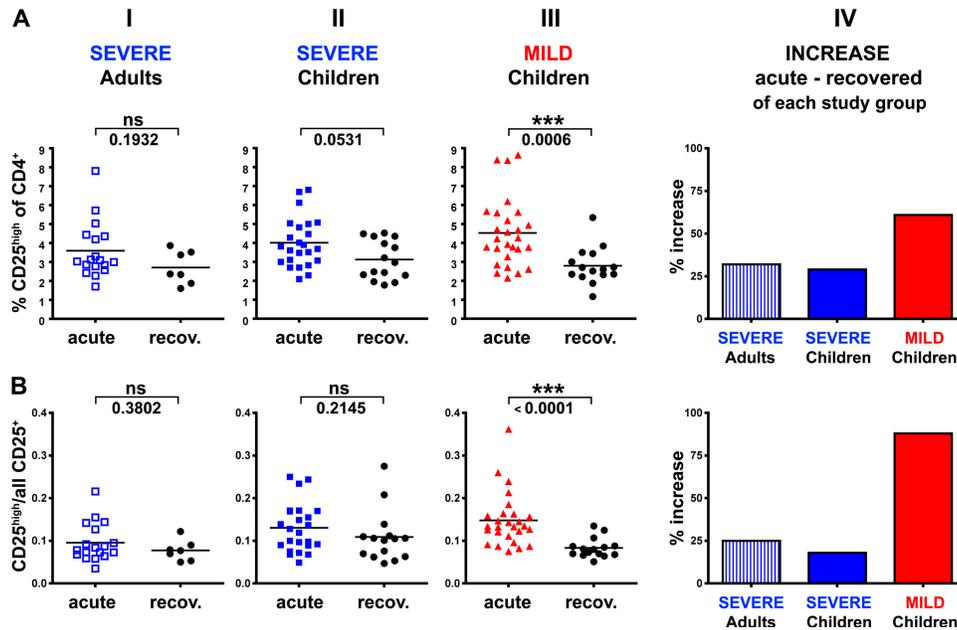


Figure 5. Increased T reg cell frequencies and T reg cell/effector T cell ratios in acute dengue infection. Patients were grouped based on severity and age (as indicated in I–III). (A) The T reg cell frequencies are given as a percentage of the CD25^{high} cells of CD4⁺ cells. (B) T reg cell/

effector T cell ratios are given. Differences of frequencies and ratios between the acute and recovered states are summarized in A (IV) and B (IV). Horizontal bars represent mean values.

T reg cells to tissue sites of DV replication in severely ill patients, current evidence suggests that the major site of DV replication is in the blood. Because the major site of both virus replication and disease pathogenesis (caused by plasma leakage) is the vasculature, our findings of an altered balance between T reg cell and effector T cell function in the blood of severely ill dengue patients are likely to have relevance to disease pathogenesis.

Frequencies of T reg cells have been analyzed in other infectious diseases, but little is known about their numbers and function in acute viral infections in man. An expansion has been observed in acute mouse herpes simplex virus infection (22). With the identification of FoxP3 and our observation of reduced CD4 expression, T reg cells and activated T cells can clearly be distinguished, and more information is expected.

For the first time, we describe in this study the role of T reg cells in a nonpersistent viral infection. The balance between regulatory and effector functions determines the outcome of disease. In most of the infections examined so far, persistence of the pathogen seems to be the price to limit immunopathology (12). The DV is cleared quickly within 7 d of illness onset. However, the host may pay with substantial immunopathology.

Beside T cells, T reg cells regulate innate immune and B cells (Vanitha, D.J., and B.T. Rouse, personal communication; reference 23). This is particularly interesting in dengue, as T reg cells could influence antibody-dependent enhancement and innate immune cell-driven vasoactive cytokine release. Our data suggest a possible influence of T reg cells on monocyte activity, but further studies are needed.

In this study, we provide the first description of T reg cells in dengue infection. Although they proliferate in acute

disease and appear to function normally, we suggest that a relatively inadequate expansion is associated with severe dengue disease. Therefore, we can add T reg cells to the list of factors influencing the outcome of dengue infection. The relative contribution of all different components should be further analyzed to shed more light on dengue pathogenesis. This will be especially important for the rational design of a DV vaccine that can provide effective protection against all four serotypes without contributing to immunopathology.

MATERIALS AND METHODS

Study design and patients. 89 serologically confirmed (24) dengue patients were enrolled at The Hospital for Tropical Diseases and grouped based on age. Pediatric patients (<15 yr) were further subdivided by severity (Table S1). Patients were admitted on days 1–10 of illness. Blood samples were collected on admission, at discharge, and as follow-up samples weeks to several months after recovery. As not all time points were available for every patient, compared samples are not necessarily matched. Dengue specificity of T reg cell function was determined using acute samples of an additional 31 dengue-infected adults. Study protocols were approved by the Scientific and Ethical Committee at The Hospital for Tropical Diseases and the Oxford Tropical Research Ethical Committee. Written informed consent was obtained from the patients or their legal guardians.

Isolation of cells. Subpopulations of Ficoll-Histopaque-isolated PBMCs were sorted with magnetic beads, as no FACS sorter was available in Vietnam. CD4⁺ cells were negatively isolated with a CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). The purity was $69.08 \pm 21.54\%$. Isolations with low purity were obtained from acute samples and contained erythrocytes. However, $95.18 \pm 2.27\%$ of all nonerythrocyte cells were CD4⁺. CD25⁺ cells were depleted as well as isolated with Dynabead CD25 (Dyna) and detached with Detachabead reagent. Purities of the nonerythrocyte CD25⁻ and CD25⁺ fractions were $92.7 \pm 9.48\%$ and $87.9 \pm 11.58\%$, respectively.

In vitro coculture proliferation assay. 5×10^3 CFSE-labeled CD4⁺CD25⁻ cells were incubated in RPMI/10% human serum with the same amount or with 2.5×10^3 CD4⁺CD25⁺ or CD4⁺CD25⁻ cells in anti-CD3-coated round-bottom 96-well plates (1 μ g/ml OKT3; eBioscience). 3×10^4 mitomycin-C-treated allogeneic EBV-immortalized B cells were added to each well as APCs. After 5 d, proliferation was analyzed by FACS analysis.

IFN- γ ELISPOT. ELISPOT was performed as described previously (24). For activation, a pool of 124 peptides of the DV-4 NS3 protein (15 mers; 10-mer overlap; 1.5 μ M each), 10 μ g/ml of inactivated DV-2 (BiosPacific), or a pool of irrelevant peptides (*Mycobacterium tuberculosis* ESAT6) were used. Responses higher than four times the background were considered positive. Cells and culture media were incubated for another 48 h before analyzing TNF- α in the supernatant.

Monocyte coculture assay. CD14⁺ monocytes were sorted (CD14 beads; Miltenyi Biotec). 10^5 monocytes and 10^5 CD25⁻ or T reg cells were cultured in 0.2% gelatine-coated round-bottom 96-well plates with or without DV-4 NS3 peptide pool. After 40 h, CD14⁺ cells were sorted again and cultured with or without inactivated DV-2 that was preincubated with autologous plasma to coat virus particles with antibodies. Supernatants were analyzed for IL-6 after 48 h.

Flow cytometry. 150 μ l of blood was incubated ex vivo with combinations of four antibodies (BD Biosciences) targeting CD4, 8, 25, 45RO, 62L, 69, 95, and CTLA-4. Suitable isotype controls were performed. Intracellular CTLA-4 was detected in parallel to FoxP3. Cells were analyzed with a flow cytometry system (FACSCalibur; BD Biosciences) and FlowJo software (Tree Star).

FoxP3 analysis. FoxP3 analysis was performed by flow cytometry using the FoxP3 staining set (clone PCH101; eBioscience).

Cytokine analysis. The levels of secreted IL-10, TNF- α , and IL-6 were determined in culture supernatants using a Bio-Plex Cytokine Assay (Bio-Rad Laboratories).

Statistical analysis. Statistical significance was calculated with nonparametric unpaired (Mann-Whitney) or paired (ELISPOT and monocyte assays) Student's *t* tests using Prism 4 software (GraphPad). *P*-values of <0.05 were regarded as significant.

Online supplemental material. Table S1 presents summary characteristics of serologically confirmed dengue cases. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061381/DC1>.

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